The Saudi Dental Journal (2010) **22**, 27–34

King Saud University

The Saudi Dental Journal

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ORIGINAL ARTICLE

Differential expression of c-kit and CD43 in histological subtypes of adenoid cystic carcinoma of salivary gland

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Received 6 January 2009; revised 22 March 2009; accepted 23 May 2009
Available online 4 January 2010

KEYWORDS

Adenoid cystic carcinoma (ACC);
Salivary gland;
Immunohistochemistry;
c-kit;
CD43

Abstract Adenoid cystic carcinoma (ACC) of the salivary gland is characterized by a prolonged but inevitably unfavorable clinical course. Recent studies have suggested that the transmembrane tyrosine kinase receptor, c-kit proto-oncogene is involved in ACC pathogenesis. CD43 is a sialoglycoprotein that is typically expressed by hematopoietic cells and their derivative neoplasms, although positivity in epithelial tumors has only been recognized recently.

Objective: The aim of this study was to evaluate c-kit and CD43 immunoreactivity in ACCs and to compare the extent of their expression in various histologically defined subgroups of ACC, and their probable involvement in ACC pathogenesis.

Study design: Formalin-fixed paraffin-embedded sections from 35 ACCs were immunostained for c-kit and CD43 using monoclonal antibodies.

Results: Cytoplasmic and membranous c-kit immunoreactivity was detected in 25/35 ACCs (71.4%) with strong immunostaining observed in solid pattern of ACC. Cytoplasmic and membranous CD43 immunoreactivity was detected in 18/35 (51.4%) of ACCs with strong immunostaining seen in the cribriform pattern.

Conclusions: These results suggested that c-kit could be used as a prognostic marker for ACC and specific c-kit tyrosine kinase inhibitors such as *imatinib*, might be used in future therapeutic approaches against subgroups of ACC. CD43 appears to be preferentially expressed in salivary gland ACCs. Its expression decreased with cellular dedifferentiation and there was an inverse relationship between immunoexpression of c-kit and CD43 among ACC of salivary gland.

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doi:10.1016/j.sdentj.2009.12.001

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1. Introduction

Adenoid cystic carcinoma (ACC) is characterized by a slow, but locally aggressive growth pattern along nerves and blood vessels irreversibly destructing adjacent and surrounding tissues of the head and neck region. Even though the 5-year overall survival is more than 70%, long-term outcome is much less favorable declining to less than 10% after 20 years because of

late local recurrences and distant metastases (Kokemueller et al., 2004).

ACC shows three distinct histological differentiation types with the cribriform subtype exhibiting islands of monomorphic cells with punched-out spaces and the tubular subtype showing narrow ductal structures within a fibrous stroma. The latter is supposed to have a better prognosis than the less differentiated solid subtype, which is composed of basaloid tumor cells with nuclear pleomorphism and high mitotic activity (Fordice et al., 1999). Specific cytogenetic aberrations involved in initiation and progression of these histologically defined ACC subtypes are infrequently found and only poorly described (Stallmach et al., 2002).

The evolution of ACC is unpredictable in the individual patient because of the lack of strong prognostic indicators. Surgery and postoperative radiation remains the mainstay of therapy. Although various chemotherapy regimens have been tried, none has proved to be effective in the treatment of ACC (Jeng et al., 2000).

The transmembrane tyrosine kinase receptor c-kit (CD117) is a 145- to 165 Kd proto-oncogene that structurally belongs to a family of receptors that include those for colony-stimulating factor-1 (CSF-1) and platelet derived growth factor (PDGF) (Vliagoftis et al., 1997; Jeng et al., 2000). After binding to the ligand (stem cell factor), dimerization, and phosphorylation, the receptor tyrosine kinase (c-kit) subsequently activates a signaling cascade that regulates cell growth and development of multiple hematopoietic cell lineages (Fletcher, 2004). In addition to hematopoiesis, c-kit has been shown to play a role in normal migration and development of germ cells and melanocytes (Funasaka et al., 1992; Jeng et al., 2000).

The c-kit protein is required for normal hematopoiesis, melanogenesis, and gametogenesis. Thus c-kit gene product is expressed in several normal cell types including mast cells, intestinal cells of Cajal, melanocytes, and epithelial cells of breast (Edwards et al., 2003). Overexpression of the c-kit tyrosine kinase receptor occurs in a number of neoplasms, including germ cell tumors (Izquierdo et al., 1995), gastrointestinal stromal cell tumors (GISTs) (Sarlomo-Rikala et al., 1998), malignant melanoma (Guerriere-Kovach et al., 2004), leukemias, endometrial carcinomas, papillary and follicular thyroid carcinomas, and ACC (Jeng et al., 2000; Edwards et al., 2003; Chandan et al., 2004; Freier et al., 2005; Aslan et al., 2005; Andreadis et al., 2006).

c-kit is a target of the tyrosine kinase inhibitor *imatinib mesylate* (Gleevec™), which showed significant treatment response in patients with chronic myelogenous leukaemia (CML) (O'Brien et al., 2003) and advanced c-kit-positive GIST (Verweij et al., 2004).

CD43, also known as leukosialin, sialophorin, and gp115, is a transmembrane sialoglycoprotein expressed on the cell surface of most hematopoietically-derived cells, including T lymphocytes, granulocytes, monocytes, and platelets. Two isoforms of CD43 exist that differ both in antigenicity and molecular weight: the first form possesses an affinity for the thymocyte/lymphocyte/monocyte cell lines (115-kDa form); the second form favors the neutrophil/platelet cell lines (135-kDaform) (Pimenidou et al., 2004). The molecular configuration of CD43 is similar to that of mucin, with multiple sialylated O-glycan sites and a single N-linked glycan site (Cruz-Munõz et al., 2003). Of note, the different isoforms of CD43 appear to be determined by minor alterations in the glycosylation pattern of this glycoprotein (Santana et al., 2000).

CD43 has been demonstrated to be a multifunctional protein with often paradoxical roles in a variety of cellular processes. Its involvement in cellular adhesion events is directly related to post-translational modifications of the extracellular domain, such as high level of glycosylation and heavy sialylation; these modifications appear to facilitate cell–cell repulsion or promote cell–cell adhesion, respectively (Cruz-Munõz et al., 2003; Pimenidou et al., 2004). In addition, CD43 participates in a complex signaling pathway that results in recruitment of several signaling proteins, activation of protein kinase C (PKC), AP-1, and NFκB, and direct induction of various genes (Santana et al., 2000), ultimately culminating in activation of T lymphocytes and natural killer (NK) cells (Santana et al., 2000; Cruz-Munõz et al., 2003).

CD43 expression can be seen on a number of neoplasms, primarily of hematopoietic origin. Positive reactivity has been demonstrated in a majority of T cells, mantle cell, small lymphocytic cells, and Burkitt's lymphoma with less frequent expression identified in nodal and extranodal marginal zone lymphomas (Lai et al., 1999). Aberrant expression of CD43 has also been noted in plasmacytomas (Petruch et al., 1992; Shin et al., 2001). Evidence also suggests a role for CD43 in epithelial neoplasms. Study has demonstrated CD43 expression in the colon adenocarcinoma cell line COLO 205 (Baeckström, 1997).

Seethala et al. (2004) documented aberrant expression of CD43 in adenoid cystic carcinomas of salivary and mammary glands origin. They reported preferential immunoreactivity of CD43 in adenoid cystic carcinomas compared to non-adenoid cystic carcinoma tumors included in their study.

2. Materials and methods

A total of 35 adenoid cystic carcinomas of the salivary gland were retrieved from the files of Department of Oral and Maxillofacial Pathobiology, Graduate School of Medical Sciences, Hiroshima University. Representative hematoxylin and eosin-stained sections of all the tumors were reviewed to confirm the tumor type and to assign the differentiation grade (12 cribriform, 14 tubular and nine solid variants). Five fresh normal salivary gland tissues serving as controls were collected from sialadenectomy specimens and processed as usual for formalin-embedded paraffin blocks for hematoxylin and eosin as well as immunostaining.

For immunohistochemistry (IHC), Four micron serial sections were performed from each formalin fixed paraffin-embedded tissue blocks, mounted on charged slides and dried. To enhance immunoreactivity, sections were subjected to microwave heat treatment as follows: the slides were first deparaffinized, dehydrated in graded ethanol concentrations, and incubated with 0.6% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. After rinsing with water, the slides were placed in a glass dish filled with 10 mmol/L sodium citrate buffer, pH 6.0. Tissue sections were boiled in a microwave oven twice for 5 min each to enhance immunoreactivity. The slides were allowed to cool and rinsed with phosphate-buffered saline (PBS), pH 7.2. The immunohistochemical staining was done according to the manufacturer's instructions using. Anti c-kit (poly clonal, DAKO

Corp., Carpinteria, CA) at a dilution 1:100 with heat-induced epitope retrieval on a NexES instrument (Ventana Medical System, Tucson, AZ) and Anti CD-43 (clone L 60, Venta Medical system, Inc, Tucson, AZ) at a 1:50 dilution . Detection was carried out using DAKO universal kit (Glostrup, Denmark). Slides were washed in PBS for 5 min and incubated with secondary antiserum that was biotinylated goat serum covalent to rabbit and mouse serum for 30 min. Sections were then washed for 5 min in PBS followed by development of antigen–antibody visualization by diaminobenzidine [DAB in PBS containing 40% hydrogen peroxide. Sections were washed under running tap water for 10 min lightly, counter stained with Mayer's haematoxylin and mounted. Negative controls were carried out on consecutive sections, using either an isotype antibody or omission of the primary anti-body resulting in no detectable staining.

3. Evaluation of immunostaining

Tumor cells with unequivocal staining of the cytoplasm or the membrane were considered positive. Cases were divided into three categories based on the percentage of positive cells and intensity of c-kit and CD43 expression.

The immunostained sections were examined using light microscope to assess the prevalence of positive cases and the localization of immunostaining within the tissues. The image analysis was performed using a computer system, Germany (software Leica Qwin) consisting of color video camera, color monitor, hard disc of hp personal computer connected to the microscope, Oral Pathology Department, Faculty of Dental Medicine for Girls, Al-Azhar University. Tumor cells with unequivocal staining of the cytoplasm or the membrane were considered positive for both c-kit and CD-43. The percentage of positive cells was measured in the form of an area and area percent inside a standard measuring frame of area 11434.9 μm^2 per 10 fields using a magnification (40×) by light microscopy then transferred to the monitor. These areas were masked by a blue binary color using the software computer system for measurement. Mean values were obtained for the whole specimens.

The percentage of positive cells was scored as follows: 0 (0–19%), 1 (20–39%), 2 (40–59%), and 3 (60–100%). The intensity of expression was assessed according to the staining intensity as grade 1 (mild), 2 (moderate), 3 (strong). The percentage score and the intensity score were summed up to obtain the total score, which was graded as follows: mild for 1, moderate for 2, and strong for 3, according to Lim et al. (2003).

4. Statistical evaluation

Quantitative data of the image analyzer were first summarized and presented as means and standard deviation values. Analysis of variance (ANOVA) was used to compare between means of the three groups. Duncan's post-hoc test was used to determine significant differences between the means when ANOVA test result was significant. Qualitative data were presented as frequencies and percentages. Chi-square (χ^2) test was used to compare between the groups. The significance level was set at $P \leqslant 0.05$. Statistical analysis was performed with SPSS15.0® (Statistical Package for social sciences) for Windows.

5. Results

The ACC comprised of 35 cases, 13 men and 22 women with a median age of 62 years (range: 50–81 years). The tumor sites include 9 lesions from major salivary gland (2 parotid, 6 submandibular, 1 sublingual) and 26 lesions from minor salivary gland (14 palate, 2 tongue, 1 lower lip, 8 floor of the mouth (FOM), 1 retromolar), metastasis was reported in 6 cases, (Table 1). All morphologic patterns classically described in ACC, including cribriform, tubular, and solid, were presented. Six cases showed metastasis, of which 4 cases were of solid pattern and 2 cases were tubular.

The five normal salivary gland tissue specimens revealed negative immunostaining for both c-kit and CD43. Considering c-kit, it was expressed by 25/35 cases of ACC (71.4%) with cribriform pattern 7/12 (58.3%), tubular 10/14 (71.4%), solid pattern 8/9 (88.8%) (Table 2), whereas, CD43 was expressed by 18/35 cases (51.4%) exhibited a positive staining for CD43 with cribriform pattern 10/12 (83.3%), tubular pattern 5/14 (27.7%), solid pattern 3/9 (33.3%), (Table 3).

Distribution of c-kit immunostaining was observed primarily in the cytoplasm although when cytoplasmic staining was strong, a membranous pattern of immunostaining was also observed. The tubular and cribriform patterns showed c-kit expression in the inner luminal cell layers of the tumor (Figs. 1A and 3A), whereas the solid pattern showed c-kit expression in all neoplastic cells (Fig. 2A). For CD43, cytoplasmic and membranous immunoreactivity was detected.

The immunostaining for c-kit varied in intensity from mild to strong among different ACC subtypes. The solid pattern showed strong immunopositivity (8/8), whereas tubular pattern exhibited mild (1/10), moderate (8/10), strong (1/10) immunopositivity. The cribriform patterns showed mild immunopositivity (7/7). The four solid pattern that metastasized revealed strong immunostaining, while the two tubular cases showed negative staining.

Cytoplasmic CD43 immunostaining varied from mild to strong among the ACC subgroups. Notably, there was a trend towards more tumors without any immunostaining for CD43 in solid ACC than in cribriform/tubular ACC. Tumors exhibiting a strong immunostaining were only found in cribriform and tubular compared to solid subtypes. The cribriform pattern showed moderate immunostaining in (1/10 cases) and strong immunostaining in (9/10 cases) (Fig. 1B), the tubular pattern revealed moderate immunostaining in (4/5 cases) and strong immunostaining in (1/5 cases) and mild immunostaining (Fig. 3B), whereas, the solid variant showed mild immunostaining (2/3 cases) and moderate immunostaining (1/3 cases) (Fig. 2B). Among the four solid pattern cases that metastasized, 3 cases revealed negative immunostaining and one case with mild immunostaining, while the two tubular pattern cases revealed negative immunostaining.

For c-Kit, solid ACC showed statistically significant highest mean area percent. There was no statistically significant difference between cribriform and tubular ACC which showed the lowest mean value. There was no statistically significant difference between mean optical densities of the three groups (Table 4) (Figs. 4 and 5).

For CD43, cribriform ACC showed the statistically significant highest mean area percent. This was followed by tubular ACC. Solid ACC showed significantly lowest mean. Cribri-

Table 1 Clinicopathological features and immunohistochemical results.

Case number	Age	Sex	Location	Pattern	Metastasis	C-kit	CD43
1	40	M	Palate	Tubular	–	+	–
2	76	M	Lower lip	Tubular	–	+	–
3	55	F	SM	Cribriform	–	+	+
4	78	F	FOM	Cribriform	–	–	+
5	65	F	FOM	Cribriform	–	+	+
6	81	F	FOM	Cribriform	–	–	–
7	77	F	Tongue	Tubular	–	+	–
8	79	M	Palate	Tubular	–	+	+
9	46	F	FOM	Cribriform	–	–	+
10	79	M	Palate	Solid	–	+	–
11	40	M	Palate	Tubular	–	–	+
12	76	M	Palate	Cribriform	–	+	+
13	84	F	FOM	Tubular	–	+	–
14	74	F	SM	Cribriform	–	+	–
15	65	M	SM	Solid	–	+	+
16	79	F	SM	Tubular	–	+	–
17	74	M	SM	Cribriform	–	–	+
18	37	F	FOM	Solid	+	+	–
19	74	F	Palate	Solid	+	+	–
20	48	F	Retromolar	Tubular	+	–	–
21	63	F	FOM	Tubular	+	–	–
22	45	F	Palate	Cribriform	–	+	+
23	65	F	Palate	Tubular	–	+	–
24	45	F	Palate	Cribriform	–	–	+
25	48	M	Palate	Solid	–	+	–
26	64	F	Palate	Tubular	–	+	+
27	58	M	Parotid	Tubular	–	+	+
28	48	F	Tongue	Tubular	–	–	–
29	48	F	SLG	Solid	–	–	–
30	73	M	Palate	Solid	–	+	+
31	30	F	Palate	Solid	+	+	+
32	70	F	SM	Solid	+	+	–
33	46	F	FOM	Cribriform	–	+	+
34	65	M	Parotid	Tubular	–	+	+
35	75	M	Palate	Cribriform	–	+	+

F = female, M = male, SM = submandibular, SLG = sublingual gland, FOM = floor of mouth.

Table 2 Summary of IHC results of c-kit in ACC by histologic subtype.

IHC staining ACC subtype	Number of cases	Negative	Mild	Moderate	Strong	Total positive cells (%)
Cribriform	12	5	7	0	0	7 (58.3%)
Tubular	14	4	1	8	1	10 (71.4%)
Solid	9	1	0	0	8	8 (88.8%)
All subtypes	35	10	8	8	9	25 (71.4%)

Table 3 Summary of IHC results of CD43 in ACC by histologic subtype.

IHC staining ACC subtype	Number of cases	Negative	Mild	Moderate	Strong	Total positive cells (%)
Cribriform	12	2	0	1	9	10 (83.3%)
Tubular	14	9	0	4	1	5 (27.7%)
Solid	9	6	2	1	0	3 (33.3%)
All subtypes	35	17	2	6	10	18 (51.4%)

form ACC showed the statistically significant highest mean optical density. This was followed by tubular ACC. Solid ACC showed the significantly lowest mean (Table 4) (Figs. 4 and 5).

6. Discussion

ACC is an extremely unpredictable tumor because of slow growth, late metastases, and lack of strong prognostic mark-

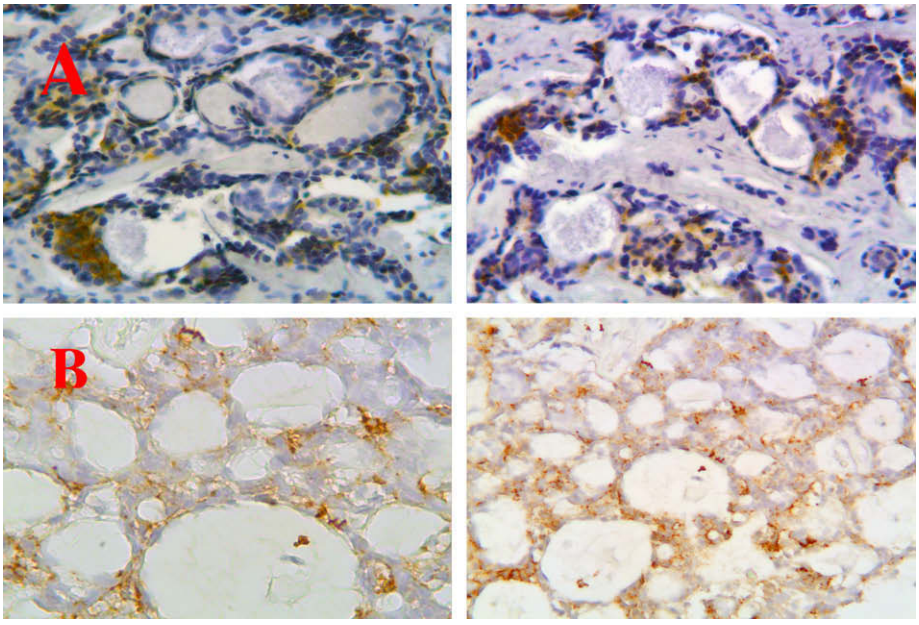

Figure 1 Photomicrographs of (A). Cytoplasmic c-kit expression in cribriform pattern of ACC, predominantly in luminal cells (B). Cytoplasmic and membranous CD43 expression in cribriform ACC (original magnification 200×).

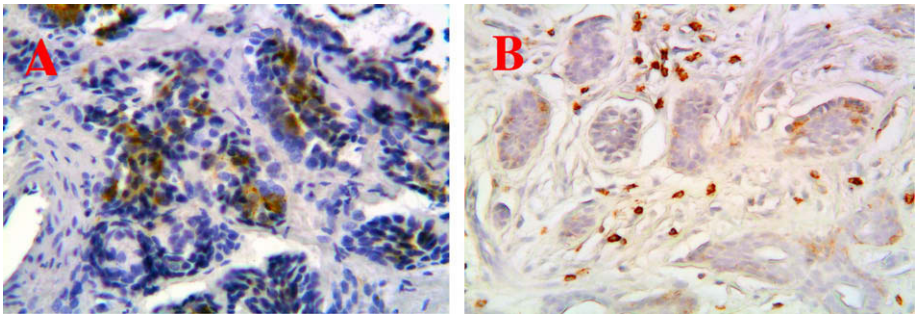

Figure 2 Photomicrographs of (A). Cytoplasmic c-kit expression in solid form of ACC, predominantly in all neoplastic cells (B). Cytoplasmic CD43 expression in solid form ACC, (original magnification 200×).

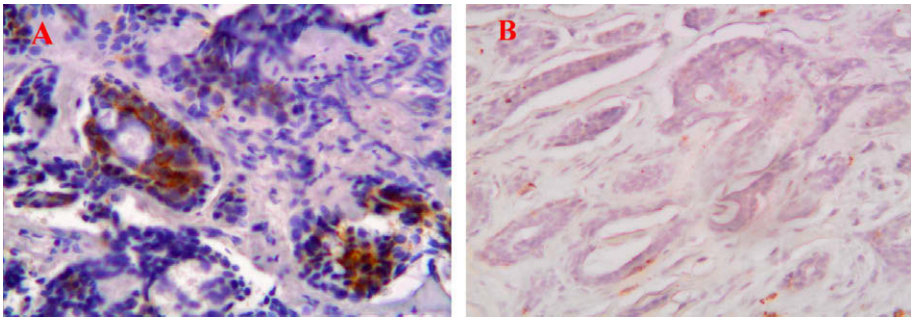

Figure 3 Photomicrographs of (A). Cytoplasmic and membranous c-kit expression in tubular pattern of ACC, predominantly in luminal cells (B). Cytoplasmic CD43 expression in tubular pattern of ACC (original magnification 200×).

ers. In this study, there was an attempt to find a correlation between c-kit and CD43 immunoexpression and tumor differentiation of ACCs. As regard c-kit, a reverse relationship with the degree of tumor differentiation was detected, while, CD43 showed a direct relationship as proved by highly significant statistical results.

The negative immunostaining of c-kit in normal salivary gland tissues was in accordance with result of Chandan et al.

Table 4 Statistical results of mean Area percent and optical density for c-kit and CD43 among the ACC subgroup.

		Cribriform		Tubular		Solid		*P*-value
		Mean	SD	Mean	SD	Mean	SD	
c-Kit	Area percent	2.01[b]	0.9	3.31[b]	1.1	6.51[a]	1.4	0.027*
	Optical density	68.6	5.9	72.6	8.3	74.1	9.1	0.164
CD43	Area percent	23.04[a]	1.2	9.68[b]	1.7	4.53[c]	1	<0.001*
	Optical density	70.2[a]	8.4	62.1[b]	6.5	53.3[c]	4.3	<0.001*

* Significant at $P \leqslant 0.05$, means with different letters are statistically significantly different according to Duncan's test.

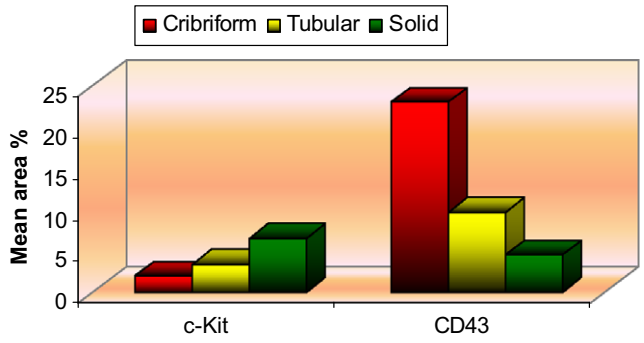

Figure 4 Mean area percentage of c-kit and CD43 immunoexpression.

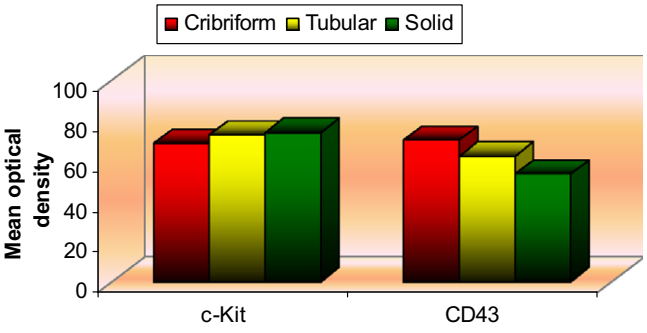

Figure 5 Mean optical density of c-kit and CD43 immunoexpression.

(2004). Which suggested that c-kit protooncogene may have no role in salivary gland function rather initiation of salivary gland tumors.

In this study, solid pattern of ACC showed the statistically significant highest mean area percent (*P* value = 0.027*) for c-kit. There was no statistically significant difference between cribriform and tubular ACC which showed the statistically significant lowest means. There was no statistically significant difference between mean optical densities of the three groups (*P* value = 0.164).

The pattern of c-kit expression in ACC differed with the histology subtype. Tubular and cribriform variants primarily showed c-kit expression in the luminal cell layer. This suggested that the myoepithelial cell did not express c-kit, as the abluminal cells did not stain. This was in accordance with study of Edwards et al. (2003) and Aslan et al. (2005). Paradoxically solid variants showed expression in all cells most of which are considered modified myoepithelial cells (Ellis and Auclair, 1995). This difference in the pattern of c-kit expression in tubular and cribriform ACC, as compared with solid variants of ACC, suggest a loss of cellular heterogeneity in solid variants, with differentiation primarily along the line of the luminal cell layer, and may correlate with the worse clinical course of the solid variant of ACC seen in some studies (Cruz-Munõz et al., 2003). These were in line with finding that the strong immunopostivity detected in highly malignant solid phenotype and mild immunopostivity detected in cribriform pattern, which have clinical feature of moderate aggressiveness. These results were in accordance with study of Edwards et al. (2003), Chandan et al. (2004) and Aslan et al. (2005). Meanwhile, contradictory to these results Freier et al. (2005) found that strong immunostaining of c-kit was only found in cribriform and tubular but never in solid subtypes.

Previous studies reported that c-kit gene mutations or phosphorylation at the protein level was not detected in ACC, even though the c-kit protein was over-expressed (Jeng et al., 2000; Oliveira et al., 2003) in most tumors. These studies may suggest that c-kit tyrosine kinase may not affect the natural history of ACC.

In this study, cribriform ACC showed the statistically significant highest mean area percentage for CD43 (*P* value = <0.001*). This was followed by tubular ACC. solid ACC showed the statistically lowest mean. Cribriform ACC showed the statistically significantly highest mean optical density (*P* value = <0.001*). This was followed by tubular ACC. Solid ACC showed the significantly lowest mean. Both area percentage and optical density goes in line with each other for CD43 immunostaining.

According to present data, no expression of CD43 was observed in nonneoplastic salivary gland tissue specimens. Although weak focal staining ductal epithelial cells were observed, this immunoreactivity was below the threshold for designation of positive staining in the current study. CD43 immunostaining is more frequently found in well-differentiated tumors and might get lost during dedifferentiation of ACC, indicating that different molecular pathways are involved in the formation of histological ACC subtypes.

Both cribriform and tubular pattern showed strong to moderate immunostaining for CD43, while, solid pattern revealed mild to moderate CD43 immunostaining. This indicated that CD43 is important in initiation of ACC rather than progression. These results were in accordance with results of Seethala et al. (2008) as they found that the few high-grade, angioinvasive, ACC did not seem to express CD43. Contrary to these results, Woo et al. (2006) found that there were no difference in immunostaining intensity observed when reactivity was assessed according to ACC subtypes.

CD43 is a recognized receptor of intercellular adhesion molecule-1 (ICAM-1, CD54), Rosenstein et al. (1991) a complex, multifunctional molecule with major roles in trafficking of inflammatory cells and in antigen presentation to T lymphocytes ICAM-1 is widely expressed in cells of both hematopoietic and non-hematopoietic origin, including epithelial, endothelial, and fibroblastic cells.

Previous study by Ziprin et al. (2003) reported that ICAM-1 expressed by mesothelial cells enhanced colorectal adenocarcinoma cell attachment to the peritoneum. The same group later demonstrated that cells expressing CD43, including three colorectal adenocarcinoma cell lines, a pancreatic adenocarcinoma cell line, and an ovarian carcinoma cell line, that had been previously incubated with anti-CD43 antibodies and anti-ICAM-1 antibodies, exhibited reduced adhesion to a mesothelial cell monolayer Ziprin et al. (2004). These authors postulated that the interaction between ICAM-1 and its ligand CD43 was likely implicated in the development of peritoneal metastases of various solid epithelial tumors Ziprin et al. (2004). Similarly, it is possible that the CD43-ICAM-1 interaction participates in the unique capacity of ACCs to undergo distant metastases, a characteristic not shared by other salivary gland tumors.

In the current study, there was an inverse relationship between immunoexpression of c-kit and CD43 among ACC of salivary gland. The uniform strong expression of c-kit seen in this and previous studies of ACC suggested that c-kit may be used as a prognostic marker in ACC. It also, suggested a potential role for c-kit inhibitors such as imatinib mesylate (GleevecTM), formerly STI-571, in the treatment of patients with advanced ACC. CD43 appears to be preferentially expressed in salivary gland ACC, its expression decreases with cellular dedifferentiation and there was an inverse relation between immunoexpression of c-kit and CD43 among ACC of salivary gland.

Acknowledgements

Deep appreciation is due to Prof Takata Takashi Dean of Graduate School of Medical Sciences Hiroshima University for his continuous help and support during the study in Japan. We gratefully acknowledge for Dr. Ogawa I, Department of Oral and Maxillofacial Pathobiology, Graduate School of Biomedical Sciences, Hiroshima University for her valuable assistance in this study.

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